REPROGRAMMING NUCLEAR FUNCTION WITH SOMATIC CELL CYTOPLASM

CROSS-REFERENCE TO RELATED PATENT APPLICATION

This application claims the benefit, pursuant to 35 U.S.C. §119(e), of provisional U.S. Patent Application Serial No. 60/464,227, filed April 21, 2003, the disclosure of which is hereby incorporated herein in its entirety by reference. This application also claims the benefit of priority to co-pending PCT International Patent Application, PCT/US04/001375, filed on January 20, 2004.

FIELD OF THE INVENTION

In general, the invention features methods for reprogramming the differentiated state of somatic cells thereby transforming cells committed to a particular differentiated state into cells with a different state of differentiation. It also features methods for administering these reprogrammed cells to a mammal for the treatment or prevention of disease, and using the cells in biomedical research and drug discovery.

BACKGROUND OF THE INVENTION

Despite having essentially the same genome, different classes of somatic cells in a particular mammal have distinctive phenotypes due largely to the different combinations of genes that they express. These different expression profiles allow cells to perform certain functions, such as the secretion of a tissue-specific hormone or extracellular matrix components such as type I collagen in cartilage.

Because many diseases and injuries are caused by damage or malfunction of a particular class of cells, methods are needed to produce cells of a desired cell type

that may be used to replace these damaged cells. These replacement cells may have the same genotype as the damaged cells.

SUMMARY OF THE INVENTION

In general, the invention comprises the use of a somatic cell cytoplast or somatic cell cytoplasm to reprogram the nucleus of a different cell. Herein, "reprogramming" means that the differentiated cell nucleus expresses or does not express at least one gene as a result of the exposure thereto to the somatic cell cytoplasm. For example, the differentiated cell nucleus will express one or more genes expressed which are characteristically expressed by the somatic cell from which the somatic cell cytoplasm is derived, but which genes are not normally expressed by the differentiated cell from which the differentiated cell nucleus is derived.

Reprogramming can be accomplished by any physical means that provides for the differentiated cell nucleus and the somatic cell cytoplasm to be incubated with one another for a sufficient time and at a concentration sufficient to facilitate nuclear reprogramming to occur.

For example, in one embodiment a large cytoplast is produced which is used to (i) dilute the components of the transplanted differentiated cell nucleus that regulate the function thereof in the somatic cell cytoplasm contained therein and (ii) several divisions are allowed to take place under conditions whereby cell growth does not occur and synthetic activity is minimal. (Essentially, when large cells are made, then allowed to divide normally, they decrease in size until they attain normal cell size.)

More specifically, the nuclear reprogramming method of the invention may include the production of somatic cell cytoplasts. Somatic cell cytoplasts can be made from any desired somatic cell type. For instance, desired tissue stem cells can be propagated in culture or alternatively genetically transformed cells which divide indefinitely can be propagated in cell cultures. Another alternative is that somatic cell cytoplasm can be recovered from desired differentiated tissues, e.g., human tissues.

Sufficient numbers of these cells are obtained to allow for the production of large cytoplasts that comprise somatic cell cytoplasm. Large cells can then be

produced that contain such somatic cell cytoplasm by different means, e.g. by fusion or one or more cells together, or by inhibition of cell division.

These cytoplasts will be treated to remove or inactivate endogenous nuclei and will be transplanted with a desired differentiated cell nucleus that is to be reprogrammed, e.g. a nucleus derived from the cell of a human subject that is in need of cell therapy. Enucleation can be accomplished by methods well known in the art of nuclear transfer, e.g. by centrifugation or aspiration.

Additionally, nuclear transfer of the desired nucleus, e.g. human nucleus into the enucleated cytoplast can be achieved by known methods in the art of nuclear transfer, e.g. cell fusion or nuclear injection. Nuclear injection can be accomplished on a small scale (single cell) or on a large scale.

The transplanted nucleus may optionally be genetically modified. Genetic modification can be accomplished before or after nuclear transplantation. Genetic modification can also be accomplished by known means for introduction or one or more genetic modifications, e.g., substitution, addition, or deletions.

For example, a desired DNA modification may be introduced by microinjection, by use of a viral vector, electroporation, etc. Additionally, desired DNA may be introduced, deleted, or modified at a specific site by use of vectyors that facilitate site specific genetic modification, e.g. by homologous recombination.

Whether a particular differentiated cell nucleus has been successfully "reprogrammed" may be discerned by different methods. In one embodiment, it will be determined whether the cytoplast gene or a gene normally expressed by the somatic cell from which the cytoplasm is obtained, but not normally expressed by the differentiated cell from which the nucleus is obtained, is expressed or not expressed. Detection of gene expression by reprogrammed nuclei can be effective by known methods, e.g. by DNA or RNA hybridization, detection of gene expression at the protein level by use of suitable probes, e.g. antibodies that bind the protein expressed by the particular gene, PCR detection methods, as well as other methods known in the art. Reprogramming may be partially complete, i.e., while complete reprogramming may result in the nucleus expressing the same gene profile as the somatic cell from which the somatic cell cytoplasm used for reprogramming is obtained, or, for example if reprogramming is partial there may be only certain genes that are normally expressed or not expressed by the somatic cell "turned on" or "turned off" as a result of nuclear reprogramming. This will vary dependent upon

several factors including but not limited to, length of exposure time of nucleus to somatic cell cytoplasm, cell source of somatic cell cytoplasm, amount of somatic cell cytoplasm, concentration of somatic cell cytoplasm in the cytoplast, whether nucleus and somatic cell cytoplasm are derived from the same or similar species, e.g. both a human nucleus and human somatic cell cytoplasm used for reprogramming, and the age of the differentiated cell nucleus that is reprogrammed.

The present invention provides methods for altering the differentiated state of cells. In particular, these methods involve incubating a permeabilized cell, nucleus or chromatin mass from a donor cell with an extract under conditions that allow nuclear components such as transcription factors to be added to, or removed from, the nucleus or chromatin mass. The added transcription factors promote the expression of mRNA or protein molecules found in cells of the desired cell type, and the removed transcription factors would otherwise promote expression of mRNA or protein molecules found in the donor cell. If desired, the chromatin mass may then be incubated in an interphase extract to reform a nucleus that encapsulates desired factors from either extract. Then, the nucleus or chromatin mass is inserted into a recipient cell or cytoplast, forming a reprogrammed cell of the desired cell type. In a related method, a permeabilized cell is incubated with an extract to allow the addition or removal of factors from the cell, and then the plasma membrane of the permeabilized cell is resealed to enclose the desired factors and restore the membrane integrity of the cell.

Accordingly, in a first aspect, the invention provides a method of reprogramming a cell. This method involves incubating a nucleus with an extract, or cytoplasm from a somatic cell, under conditions that allow the removal of a factor from the nucleus or the addition of a factor to the nucleus. Then the nucleus or a chromatin mass formed from incubation of the nucleus in the extract is inserted into a recipient cell or cytoplast, thereby forming a reprogrammed cell. In one embodiment, the nucleus is incubated with an interphase extract. The nucleus can remain membrane-bounded, and the chromosomes in the nucleus do not condense during incubation with this interphase extract. In another embodiment, a chromatin mass is formed from incubation of the nucleus in a mitotic extract. This chromatin mass may then be incubated in an interphase extract under conditions that allow a nucleus to reform, and the reformed nucleus is inserted into the recipient cell or cytoplast.

In a related aspect, the invention provides another method of reprogramming a cell. This method involves incubating a chromatin mass with an extract under conditions that allow the removal of a factor from the chromatin mass or the addition of a factor to the chromatin mass. Then, the chromatin mass or a nucleus formed from incubation of the chromatin mass in an interphase extract is inserted into a recipient cell or cytoplast, thereby forming a reprogrammed cell. In one embodiment, the chromatin mass is generated by incubating a nucleus from a donor cell in a detergent and salt solution, in a protein kinase solution, or in a mitotic extract in the presence or absence of an antibody to NuMA. In another embodiment, the chromatin mass is isolated from mitotic cells.

In another related aspect, the invention provides yet another method of reprogramming a cell. This method involves incubating a permeabilized cell with an extract under conditions that allow the removal of a factor from the nucleus or a chromosome of the permeabilized cell or the addition of a factor to the nucleus or the chromosome, thereby forming a reprogrammed cell. In one embodiment, the permeabilized cells is incubated with an interphase extract. The nucleus in the permeabilized cell may remain membrane-bounded, and the chromosomes in the nucleus do not condense during incubation with this interphase extract. In another embodiment, a chromatin mass is formed from incubation of the permeabilized cell in a mitotic extract. In yet another embodiment, the reprogrammed cell is incubated under conditions that allow the membrane of the reprogrammed cell to reseal. If desired, the permeabilized cell may be formed by incubating an intact cell with a detergent, such as digitonin, or a bacterial toxin, such as Streptolysin O.

These methods for reprogramming cells are useful for the generation of cells of a desired cell type, for example, for medical applications. Accordingly, the invention also provides methods for the treatment or prevention of disease in a mammal that include administering a reprogrammed cell to the mammal.

In one such method, the invention features a procedure for treating or preventing a disease, disorder, or condition in a mammal. This method involves incubating a nucleus from a donor cell with an extract or cytoplasm from a somatic cell under conditions that allow the removal of a factor from the nucleus or the addition of a factor to the nucleus. The nucleus or a chromatin mass formed from the nucleus is inserted into a recipient cell or cytoplast, thereby forming a reprogrammed cell. The reprogrammed cell is then administered to the mammal in

need of the cell type. In one embodiment, the nucleus is incubated with an interphase extract. The nucleus can remain membrane-bounded, and the chromosomes in the nucleus need not condense during incubation with this interphase extract. In another embodiment, a chromatin mass is formed from incubation of the nucleus in a mitotic extract. This chromatin mass may then be incubated in an interphase extract under conditions that allow a nucleus to be formed from the chromatin mass, and the reformed nucleus is inserted into the recipient cell or cytoplast. The donor cell can be from the mammal in need of the cell type. Examples of diseases, disorders, or conditions that may be treated or prevented include neurological disorders including Parkinsons Disease, auto-immune, immune deficiency disorders, inflammatory, endocrine disorders such as diabetes, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

In a related aspect, the invention provides another method of treating or preventing a disease, disorder, or condition in a mammal. This method involves incubating a chromatin mass from a donor cell with an extract under conditions that allow the removal of a factor from the chromatin mass or the addition of a factor to the chromatin mass. The chromatin mass or a nucleus formed from incubating the chromatin mass in an interphase extract is inserted into a recipient cell or cytoplast, thereby forming a reprogrammed cell. In one embodiment, the chromatin mass used in this method is generated by incubating a nucleus from a donor cell in a detergent and salt solution, in a protein kinase solution, or in a mitotic extract in the presence or absence of an antibody to NuMA. In another embodiment, the chromatin mass is isolated from mitotic cells. The reprogrammed cell is then administered to a mammal in need of the cell type. The donor cell can be from the recipient mammal. Examples of diseases, disorders, or conditions that may be treated or prevented include neurological disorders including Parkinsons Disease, auto-immune, immune deficiency disorders, inflammatory, endocrine disorders such as diabetes, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

In still another related aspect, the invention provides another method of treating or preventing a disease, disorder, or condition in a mammal that involves

incubating a permeabilized cell with an extract under conditions that allow the removal of a factor from the nucleus or a chromosome of the permeabilized cell or the addition of a factor to the nucleus or the chromosome. The reprogrammed cell formed from this step is administered to a mammal in need of that cell type. In one embodiment, the permeabilized cell is incubated with an interphase extract. The nucleus in the permeabilized cell can remain membrane-bounded, and the chromosomes in the nucleus need not condense during incubation with the interphase extract. In another embodiment, a chromatin mass is formed from incubation of the permeabilized cell in a mitotic extract. In yet another embodiment, the reprogrammed cell is incubated under conditions that allow the membrane of the reprogrammed cell to reseal prior to being administered to the mammal. The permeabilized cell can be from the mammal in need of that cell type. In another embodiment, the permeabilized cell is formed by incubating an intact cell with a detergent, such as digitonin, or a bacterial toxin, such as Streptolysin O. Examples of diseases, disorders, or conditions that may be treated or prevented include neurological disorders including Parkinsons Disease, auto-immune, immune deficiency disorders, inflammatory, endocrine disorders such as diabetes, kidney, bladder, cardiovascular, cancer, circulatory, digestive, hematopoeitic, and muscular diseases, disorders, and conditions. In addition, reprogrammed cells may be used for reconstructive applications, such as for repairing or replacing tissues or organs.

In various embodiments of the invention, at least 1, 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 300, or more mRNA or protein molecules are expressed in the reprogrammed cell that are not expressed in the donor or permeabilized cell. In another embodiment, the number of mRNA or protein molecules that are expressed in the reprogrammed cell, but not expressed in the donor or permeabilized cell, is between 1 and 5, 5 and 10, 10 and 25, 25 and 50, 50 and 75, 75 and 100, 100 and 150, 150 and 200, or 200 and 300, inclusive. At least 1, 5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 300, or more mRNA or protein molecules are expressed in the donor or permeabilized cell that are not expressed in the reprogrammed cell. In yet another embodiment, the number of mRNA or protein molecules that are expressed in the donor or permeabilized cell, but not expressed in the reprogrammed cell, is between 1 and 5, 5 and 10, 10 and 25, 25 and 50, 50 and 75, 75 and 100, 100 and 150, 150 and 200, and 200 and 300, inclusive. The mRNA or protein molecules can be specific for the cell type of the donor, permeabilized, or reprogrammed cell, such

that the molecules are only expressed in cells of that particular cell type. In still another embodiment, these mRNA or protein molecules are expressed in both the donor cell (*i.e.*, the donor or permeabilized starting cell) and the reprogrammed cell, but the expression levels in these cells differ by at least 2, 5, 10, or 20-fold, as measured using standard assays (see, for example, Ausubel *et al.*, Current Protocols in Molecular Biology. John Wiley & Sons, New York, 2000).

In other various embodiments, the size of the donor or permeabilized cell differs from that of the reprogrammed cell by at least 10, 20, 30, 50, 75, or 100%, as measured using standard methods. In another embodiment, the volume of cytoplasm in the donor or permeabilized cell differs from that in the reprogrammed cell by at least 10, 20, 30, 50, 75, or 100%, based on standard methods. In yet another embodiment, the reprogrammed cell has gained or lost an activity relative to the donor or permeabilized cell, such as secretion of a particular hormone, extracellular matrix component, or antibody or transcription factor.

In still other embodiments, the extract is an interphase extract, such as an extract formed from cells synchronized in one or more of the following phases of the cell cycle: G_o, G_1, S, G_2 phase or cells arrested in the cell cycle by natural differentiation in tissue, arrested by exogenous agents or by DNA damage. In another embodiment, the extract is formed from cells synchronized in mitosis or from unsynchronized cells. The extract can be from the cell type one wishes the donor or permeabilized cell to become. The donor or permeabilized cell can be an interphase or mitotic somatic cell. In another embodiment, the extract is modified by the enrichment or depletion of a factor, such as a DNA methyltransferase, histone deacetylase, histone, nuclear lamin, transcription factor, activator, repressor, growth factor, hormone, or cytokine. In one embodiment, the chromatin mass or nucleus is purified from the extract prior to insertion into the recipient cell or cytoplast, or the reprogrammed cell is purified prior to administration into the mammal. In one embodiment, the donor or permeabilized cell is haploid (DNA content of n), diploid (2n), or tetraploid (4n), and the recipient cell is hypodiploid (DNA content of less than 2n), haploid, or enucleated.

Donor cells, permeabilized cells, recipient cells, reprogrammed cells, and sources of cytoplasts include differentiated cells, such as epithelial cells, neural cells, including dopaminergic neurons, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-cells, T-cells, erthrocytes, macrophages,

monocytes, beta cells from pancreatic islets, fibroblasts, and muscle cells; and undifferentiated cells, such as embryonic or adult stem cells. In another embodiment, the donor or permeabilized cell is a differentiated cell, and the reprogrammed cell is a differentiated cell of another cell type. In yet another embodiment, the donor or permeabilized cell is an undifferentiated cell, and the reprogrammed cell is a differentiated cell. In still another embodiment, the donor or permeabilized cell is a differentiated cell, and the reprogrammed cell is an undifferentiated cell. If desired, an undifferentiated reprogrammed cell may be induced to differentiate into a desired cell type *in vitro* using standard methods, such as by exposure to certain growth factors, hormones, or cytokines. In another embodiment, the undifferentiated reprogrammed cell differentiates into a desired cell type *in vivo* after administration to a mammal. In yet another embodiment, the donor or permeabilized cell is a B-cell or fibroblast, and the reprogrammed cell is a T-cell. It is also contemplated that the nucleus or chromatin mass may be inserted into a recipient cell or cytoplast of the desired cell type or of the same cell type as the donor or permeabilized cell. In still another embodiment, the donor cell, permeabilized cell, recipient cell, or recipient cytoplast is from a human or non-human mammal. In yet another embodiment, the donor nucleus or chromatin mass is from a transgenic cell or mammal or contains a mutation not found in the donor cell or not found in a naturally-occurring cell.

In one embodiment, a disease-causing mutation in a regulatory region, promoter, untranslated region, or coding region of a gene in a donor nucleus or chromatin mass is modified to replace the mutant sequence with a sequence that is not associated with the disease. Alternatively, a nucleic acid is inserted into the donor nucleus or chromatin mass that includes a promoter operably-linked to a sequence of the gene that does not contain a mutation associated with a disease. The sequence of the gene can be substantially identical to that of a naturally-occurring gene that does not contain a polymorphism or mutation associated with a disease. Examples of mutations that may be rescued using these methods include mutations in the cystic fibrosis gene; mutations associated with Dunningan's disease such as the R482W, R482Q, and R584H mutations in the lamin A gene; and mutations associated with the autosomal-dominant form of Emery Dreyfus muscular dystrophy such as the R249Q, R453W, and Q6STOP mutations in the lamin A gene. In the Q6STOP mutation, the codon for Gin6 is mutated to a stop codon.

In certain embodiments, transgenic donor nuclei or chromatin masses encode heterologous MHC Class I proteins having amino acid sequences substantially identical to the sequence of an MHC Class I proteins found in the mammals to whom the reprogrammed cells will be administered for therapeutic applications. Alternatively, the donor nuclei or chromatin masses may encode MHC Class I proteins having amino acid sequences substantially identical to the sequence of MHC Class I proteins found in other mammals of the same genus or species as the recipient mammal. Reprogrammed cells that express such MHC proteins are less likely to elicit an adverse immune response when administered to the mammal. Other donor nuclei or chromatin masses are modified to express a heterologous protein that inhibits the complement pathway of the recipient mammal, such as the human complement inhibitor CD59 or the human complement regulator decay accelerating factor (h-DAF) (see, for example, Ramirez *et al.*, Transplantation 15:989-998, 2000; Costa *et al.*, Xenotransplantation 6:6-16, 1999). In yet another embodiment, the donor nucleus or chromatin mass has a mutation that reduces or eliminates the expression or activity of a galactosyltransferase, such as alpha (1,3)-galactosyltransferase (Tearle *et al.*, Transplantation 61:13-19, 1996; Sandrin, Immunol. Rev. 141:169-190, 1994; Costa *et al.*, Xenotransplantation 6:6-16, 1999). This enzyme modifies cell surface molecules with a carbohydrate that elicits an adverse immune response when cells expressing this galactose alpha (1,3)-galactose epitope are administered to humans. Thus, reprogrammed cells that have a lower level of expression of this epitope may have a lower incidence of rejection by the recipient mammal.

With respect to the therapeutic methods of the invention, it is not intended that the administration of reprogrammed cells to a mammal be limited to a particular mode of administration, dosage, or frequency of dosing; the present invention contemplates all modes of administration, including intramuscular, intravenous, intraarticular, intracranial, intralesional, subcutaneous, or any other route sufficient to provide a dose adequate to prevent or treat a disease. In one embodiment, the cells are administered to the mammal from which the donor or permeabilized cell is obtained. Alternatively, the donor or permeabilized cell may be obtained from a different donor mammal of the same or a different genus or species as the recipient mammal. Examples of donor mammals include humans, cows, sheep, big-horn sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou,

water buffalo, camels, llama, alpaca, rabbits, pigs, mice, rats, guinea pigs, hamsters, dogs, cats, and primates such as monkeys. The cells may be administered to the mammal in a single dose or multiple doses. When multiple doses are administered, the doses may be separated from one another by, for example, one week to one month. One or more growth factors, hormones, or cytokines may also be administered before, during, or after administration of the cells to further bias them towards a particular cell type. Additionally, one or more immunosuppressive agents, such as cyclosporin, may be administered to inhibit rejection of the transplanted cells. It is to be understood that, for any particular subject, specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions.

As used herein, by "chromatin mass" is meant more than one chromosome not enclosed by a membrane. In one embodiment, the chromatin mass contains all of the chromosomes of a cell. A chromatin mass containing condensed chromosomes may be formed by exposure of a nucleus to a mitotic extract, or a chromatin mass may be isolated from mitotic cells as described herein. Alternatively, a chromatin mass containing decondensed or partially condensed chromosomes may be generated by exposure of a nucleus to one of the following, as described herein; a mitotic extract in the presence of an anti-NuMA antibody, a detergent and salt solution, or a protein kinase solution.

If desired, the level of chromosome condensation may be determined using standard methods by measuring the intensity of staining with the DNA stain, DAPI. As chromosomes condense, this staining intensity increases. Thus, the staining intensity of the chromosomes may be compared to the staining intensity for decondensed chromosomes in interphase (designated 0% condensed) and maximally condensed chromosomes in mitosis (designated 100% condensed). Based on this comparison, the percent of maximal condensation may be determined. Condensed chromatin masses can be at least 50, 60, 70, 80, 90, or 100% condensed. Decondensed or partially condensed chromatin masses can be less than 50, 40, 30, 20, or 10% condensed.

By "nucleus" is meant a membrane-bounded organelle containing most or all of the DNA of a cell. The DNA is packaged into chromosomes in a decondensed

form. The membrane encapsulating the DNA can include one or two lipid bilayers or has nucleoporins.

By "donor cell" is meant a cell from which a nucleus or chromatin mass is derived.

By "cytoplast" is meant a membrane enclosed cytoplasm.

By "addition of a factor" is meant the binding of a factor to a chromosome or a component of the nuclear envelope, such as the nuclear membrane or nuclear matrix. The factor is imported into the nucleus so that it is bounded or encapsulated by the nuclear envelope. In various embodiments, the amount of factor that is bound to a chromosome or located in the nucleus increases by at least 25, 50, 75, 100, 200, or 500%.

By "removal of factor" is meant the dissociation of a factor from a chromosome or a component of the nuclear envelope, such as the nuclear membrane or nuclear matrix. The factor is exported out of the nucleus so that it is no longer bounded or encapsulated by the nuclear envelope. The amount of factor that is bound to a chromosome or located in the nucleus decreases by at least 25%.

By "enrichment or depletion of a factor" is meant the addition or removal of a naturally-occurring or recombinant factor by at least 20, 40, 60, 80, or 100% of the amount of the factor originally present in an extract. A naturally-occurring or recombinant factor that is not naturally present in the extract may be added. Factors include proteins such as DNA methyltransferases, histone deacetylases, histones, nuclear lamins, transcription factors, activators, repressors, growth factors, cytokines, and hormones; membrane vesicles; and organelles. In one embodiment, the factor is purified prior to being added to the extract, as described below. Alternatively, one of the purification methods described below may be used to remove an undesired factor from the extract.

By "purified" is meant separated from other components that naturally accompany it. A factor is substantially pure when it is at least 50%, by weight, free from proteins, antibodies, and naturally-occurring organic molecules with which it is naturally associated.

By "mutation" is meant an alteration in a naturally-occurring or reference nucleic acid sequence, such as an insertion, deletion, frameshift mutation, silent mutation, nonsense mutation, or nuisance mutation. The amino acid sequence encoded by the nucleic acid sequence can have at least one amino acid alteration

from a naturally-occurring sequence. Examples of recombinant DNA techniques for altering the genomic sequence of a cell, embryo, fetus, or mammal include inserting a DNA sequence from another organism (*e.g.*, a human) into the genome, deleting one or more DNA sequences, and introducing one or more base mutations (*e.g.*, site-directed or random mutations) into a target DNA sequence. Examples of methods for producing these modifications include retroviral insertion, artificial chromosome techniques, gene insertion, random insertion with tissue specific promoters, homologous recombination, gene targeting, transposable elements, and any other method for introducing foreign DNA. All of these techniques are well known to those skilled in the art of molecular biology (see, for example, Ausubel *et al.*, *supra*). Chromatin masses and nuclei from transgenic cells, tissues, organs, or mammals containing modified DNA may be used in the methods of the invention.

By "substantially identical" is meant having a sequence that is at least 60% identical to that of another sequence or to a naturally-occurring sequence. Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center. 1710 University Avenue, Madison, WI 53705. This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

The present invention provides a number of advantages related to the alteration of the fate of cell differentiation. For example, these methods may be generally applied to produce cells of any desired cell type. Because these methods involve incubating a nucleus, chromatin mass, or permeabilized cell in an extract to allow reprogramming, the efficiency of reprogramming may be enhanced by adding factors to the extract that facilitate reprogramming or by removing factors that inhibit reprogramming. These reprogrammed cells may be transplanted into mammals for the treatment or prevention of conditions involving damage or deficiency of a particular cell type. If desired, the reprogrammed cells may be manipulated using standard molecular biology techniques to correct a disease-causing mutation before administering the cells to a recipient mammal.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

DETAILED DESCRIPTION

We have developed novel methods of reprogramming cells by exposing them or their genetic material to a cellular extract or somatic cell cytoplasm. This reprogramming refers to decreasing or eliminating the expression of genes specific for the donor cell or increasing the expression of genes specific for another cell type. For example, we have shown that incubation of nuclei from resting T-cells, B-cells, or fibroblasts in an extract from stimulated T-cells results in migration of a T-cell specific transcription factor from the extract into the nuclei. Additionally, the reprogramming of resting T-cells also induced hyperacetylation and expression of the IL2 gene, a gene that is otherwise repressed by the nuclei. Thus, an extract may be used to alter the expression profile of the genetic material of a donor cell such that it resembles that of the cells used to prepare the extract.

The methods for reprogramming a cell that are described herein may be used to convert a cell into another cell-type that is closely related by origin or character. For example, members of the connective-tissue family, such as fibroblasts, smooth muscle cells, osteoblasts, adiopocytes, and chrondrocytes, may be interconverted using these methods. Alternatively, a cell may be converted into a desired cell type that is distantly related to the donor cell and thus shares few or no characteristics or functions with the donor cell.

The present invention relates to the use of somatic cell cytoplasm or a somatic cell cytoplast (somatic cell which has been enucleated, e.g. by centrifugation) to reprogram a nucleus derived from a differentiated cell. This differentiated cell can be of the same or different species relative to the somatic cell cytoplasm. For example, the nucleus can be that of a desired mammalian cell, e.g. embryonic, fetal or adult cell, and includes all differentiated cell types, i.e., germ cells and somatic cells. In one embodiment, the nucleus will be that of a human differentiated cell, e.g., human somatic cell. This includes all differentiated cell types, e.g. cells of endodermal, mesodermal and ectodermal origin. Non-limiting examples thereof include fibroblasts, neural cells, muscle cells, cumulus cells, skin cells, e.g. epidermal cells, keratinocytes, immune cell types, e.g. T and B lymphocytes, macrophages, digestive organ cells (e.g., stomach, intestinal), pancreatic cells, tracheal cells, cells of the eye, ear, nose, lung cells, liver cells, bone marrow derived cells, blood cells, etc. As noted, the differentiated cell nucleus, which is reprogrammed can be obtained from any age cell, and includes any

differentiated cell type. In one embodiment, the differentiated nucleus will be derived form a human differentiated cell obtained from a person in need of cell therapy. However, the differentiated cell nucleus can also be derived from other mammalian species, e.g. other primates, e.g. cynomulgus monkeys, chimpanzees, baboons, orangutans, ungulates such as sheep, bovine, goats, murines e.g. rats, mice, gerbils, hamsters, rabbits, hare, bears, horse, felines (domesticated and non-domesticated), canines (domesticated and non-domesticated), etc. As noted, the nucleus that is reprogrammed may be of the same species of different species relative to the source of the somatic cell cytoplasm used for reprogramming.

In one such reprogramming method, a nucleus from an interphase donor cell is incubated in an extract prepared from interphase cells under conditions that allow export of factors, such as transcription regulatory factors, from the nucleus and the import of factors from the extract into the nucleus. The nucleus is then inserted into a recipient cell or cytoplast, forming a reprogramming cell. The cells used to prepare the interphase extract can be the cell type one wishes the reprogrammed cell to become. Due to the different factors in the nucleus of the reprogrammed cell compared to that of the donor cell, the reprogrammed cell expresses a different set of mRNA and protein molecules and thus has a different phenotype as that of the donor cell.

In a related method, the nucleus from a donor interphase cell is incubated in a mitotic extract, a detergent and salt solution, or a protein kinase solution to promote nuclear envelope dissolution and possibly chromatin condensation, forming a chromatin mass. This nuclear envelope breakdown and chromatin condensation facilitate the release of factors from the chromatin mass. Alternatively, a chromatin mass may be isolated from a donor mitotic cell. In one embodiment of this method, the chromatin mass is inserted into a recipient cell or cytoplast of the desired cell type. After this nuclear transfer, a nucleus is reformed from the donor chromatin mass. Additionally, desired factors from the cytoplasm of the recipient cell or cytoplast migrate into the nucleus and bind the exogenous chromosomes, resulting in the expression of desired genes by the reprogrammed cell.

In another embodiment of this method, the chromatin mass is first incubated in an interphase extract as described above to further promote the release of undesirable factors from the chromatin mass and the binding of desirable factors from the interphase extract to the chromatin mass. The incubation in the interphase

extract also results in the formation of a nuclear membrane, encapsulating the chromatin mass and desired factors from the extract. The reformed nucleus is then inserted into a recipient cell or cytoplast of the desired cell type or of any other cell type.

As an alternative to isolating nuclei or chromatin masses from donor cells for subsequent incubation in a reprogramming extract, donor cells may be gently permeabilized and incubated in the extract. Permeabilization of the plasma membrane allows factors to enter and leave the cell. The cells may either be incubated in an interphase extract to allow the nucleus to remain membrane-bounded or with a mitotic extract to allow the dissolution of the nuclear membrane and formation of a chromatin mass. After incubation in the extract, the plasma membrane may be resealed, trapping desired factors from the extract inside the cell.

Reprogrammed cells generated from these methods may be used to replace cells in a mammal in need of a particular cell type. The reprogramming methods may be used to either directly produce cells of the desired cell type or to produce undifferentiated cells which may be subsequently differentiated into the desired cell type. For example, stem cells may be differentiated *in vitro* by culturing them under the appropriate conditions or differentiated *in vivo* after administration to an appropriate region in a mammal.

Examples of medical applications for these reprogrammed cells include the administration of neuronal cells to an appropriate area in the human nervous system to treat, prevent, or stabilize a neurological disease such as Alzheimer's disease, Parkinson's disease, Huntington's disease, or ALS; or a spinal cord injury. In particular, degenerating or injured neuronal cells may be replaced by the corresponding cells from a mammal. This transplantation method may also be used to treat, prevent, or stabilize autoimmune diseases including, but not limited to, insulin dependent diabetes mellitus, rheumatoid arthritis, pemphigus vulgaris, multiple sclerosis, and myasthenia gravis. In these procedures, the cells that are attacked by the recipient's own immune system may be replaced by transplanted cells. In particular, insulin-producing cells may be administered to the mammal for the treatment or prevention of diabetes, or oligodendroglial precursor cells may be transplanted for the treatment or prevention of multiple sclerosis. For the treatment of prevention of endocrine conditions, reprogrammed cells that produce a hormone, such as a growth factor, thyroid hormone, thyroid-stimulating hormone, parathyroid

hormone, steroid, serotonin, epinephrine, or norepinephrine may be administered to a mammal. Additionally, reprogrammed epithelial cells may be administered to repair damage to the lining of a body cavity or organ, such as a lung, gut, exocrine gland, or urogenital tract. It is also contemplated that reprogrammed cells may be administered to a mammal to treat damage or deficiency of cells in an organ such as the bladder, brain, esophagus, fallopian tube, heart, intestines, gall bladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, or uterus.

Reprogrammed cells may also be combined with a matrix to form a tissue or organ *in vitro* or *in vivo* that may be used to repair or replace a tissue or organ in a recipient mammal. For example, reprogrammed cells may be cultured *in vitro* in the presence of a matrix to produce a tissue or organ of the urogenital system, such as the bladder, clitoris, corpus cavermosum, kidney, testis, ureter, uretal valve, or urethra, which may then be transplanted into a mammal (Atala, Curr. Opin. Urol. 9(6):517-526, 1999). In another transplant application, synthetic blood vessels are formed *in vitro* by culturing reprogrammed cells in the presence of an appropriate matrix, and then the vessels are transplanted into a mammal for the treatment or prevention of a cardiovascular or circulatory condition. For the generation of donor cartilage or bone tissue, reprogrammed cells such as chondrocytes or osteocytes are cultured *in vitro* in the presence of a matrix under conditions that allow the formation of cartilage or bone, and then the matrix containing the donor tissue is administered to a mammal. Alternatively, a mixture of the cells and a matrix may be administered to a mammal for the formation of the desired tissue *in vivo*. Preferably, the cells are attached to the surface of the matrix or encapsulated by the matrix. Examples of matrices that may be used for the formation of donor tissues or organs include collagen matrices, carbon fibers, polyvinyl alcohol sponges, acrylateamide sponges, fibrin-thrombin gels, hyaluromic acid-based polymers, and synthetic polymer matrices containing polyanhydride, polyorthoester, polyglycolic acid, or a combination thereof (see, for example, U.S. Patent Numbers 4,846,835; 4,642,120; 5,786,217; and 5,041,138).

These methods are described further below.

EXAMPLES

EXAMPLE 1

One-step in vitro reprogramming method

In the following method for reprogramming cells, nuclei are isolated from interphase cells and incubated in an interphase reprogramming extract under conditions that allow the addition of factors from the extract to the nuclei or the removal of factors from the nuclei. Preferably, the nuclei remain membrane-bounded during this incubation. The reprogrammed nuclei are then isolated from the extract and inserted into recipient cells or cytoplasts.

Preferably, the cytoplast does not contain a nucleus, chromatin mass, or chromosome. Cytoplasts may be formed using standard procedures. For example, cytoplasts may be derived from nucleated or enucleated cells. Alternatively, cytoplasts may be generated using methods that do not require an intact cell to be used as the source of the cytoplasm or as the source of the membrane. In one such method, cytoplasts are produced by the formation of a membrane in the presence of cytoplasm under conditions that allow encapsulation of the cytoplasm by the membrane.

Isolation of nuclei

Preferably, cells from the subject who will receive the reprogrammed cells are used as the source of donor nuclei. However, cells from other members of the same species or members of a different species or genus may be used. As many as several million nuclei may be isolated from synchronized or unsynchronized cell populations in culture. The cell populations may be synchronized naturally or chemically. Preferably, at least 40, 60, 80, 90, or 100% of the cells in a population are arrested in interphase, such as in one or more of the following phases of the cell cycle: G_o, G_1, S, G_2, cells naturally arrested in the cell cycle by cell differentiation or by agents that arrest the cell cycle using standard procedures.

To accomplish this, cells may be incubated, for example, in low serum, such as 5%, 2%, or 0% serum, for 1, 2, 3, or more days to increase the percentage of cells in G_o phase. To synchronize cells in G_1, the cells may be grown to confluence as attached cells and then incubated in 0.5-1 μg/ml nocodazole (Sigma Chemicals, St. Louis, MO) for 17-20 hours, as described previously (see, for example, Collas *et al.*,

1999 and references therein). The flasks containing the attached cells are shaken vigorously by repeatedly tapping the flasks with one hand, resulting in the detachment of mitotic cells and G_1 phase doublets. The G_1 phase doublets are pairs of elongated cells at the end of the division process that are still connected by a thin bridge. Detached G_1 phase doublets may be isolated from the media based on this characteristic doublet structure. The G_1 phase doublets may remain attached or may divide into two separate cells after isolation.

To increase the percentage of cells in S phase, the cells may be cultured in the presence of aphidicolin which inhibits DNA polymerase-α and thus inhibits DNA synthesis and arrests cells in S phase. Alternatively, cells may be incubated in the presence of excess thymidine. The resulting high intracellular concentration of thymidine relative to that of other nucleotides also inhibits DNA polymerase.

Cells may be synchronized in G_2 by incubating the cells in the presence of aphidicolin to arrest them in S phase and then washing the cells three times by repeated centrifugation and resuspension in phosphate buffered saline (PBS), as described herein. The cells are then incubated for a length of time sufficient for cells to enter G_2 phase. For example, cells with a doubling time of approximately 24 hours, may be incubated for between 6 and 12 hours to allow them to enter G_2 phase. For cells with shorter or longer doubling times, the incubation time may be adjusted accordingly.

The synchronized or unsynchronized cells are harvested in PBS using standard procedures, and several washing steps are performed to transfer the cells from their original media into a hypotonic buffer (10 mM Hepes, pH 7.5, 2mM $MgCl_2$, 25 mM KCl, 1 mM DTT, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM PMSF). For example, the cells may be washed with 50 ml of PBS and pelleted by centrifugation at 500 x g for 10 minutes at 4°C. The PBS supernatant is decanted, and the pelleted cells are resuspended in 50 ml of PBS and centrifuged, as described above. After this centrifugation, the pelleted cells are resuspended in 20-50 volumes of ice-cold hypotonic buffer and centrifuged at 500 x g for 10 minutes at 4°C. The supernatant is again discarded and approximately 20 volumes of hypotonic buffer are added to the cell pellet. The cells are carefully resuspended in this buffer and incubated on ice for at least one hour, resulting in the gradual swelling of the cells.

To allow isolation of the nuclei from the cells, the cells are lysed using standard procedures. For example, 2-5 ml of the cell suspension may be transferred to a glass homogenizer and Dounce homogenized using an initial 10-20 strokes of a tight-fitting pestle. Alternatively, the cell suspension is homogenized using a motorized mixer (e.g., Ultraturrax). If desired, cell lysis may be monitored using phase contrast microscopy at 40-fold magnification. During this homogenization, the nuclei should remain intact and most or preferably all of the originally attached cytoplasmic components such as vesicles, organelles, and proteins should be released from the nuclei. If necessary, 1-20 μg/ml of the cytoskeletal inhibitors, cytochalasin B or cytochalasin D, may be added to the aforementioned hypotonic buffer to facilitate this process. Homogenization is continued as long as necessary to lyse the cells and release cytoplasmic components from the nuclei. For some cell types, as many as 100, 150, or more strokes may be required. The lysate is then transferred into a 15 ml conical tube on ice, and the cell lysis procedure is repeated with the remainder of the suspension of swollen cells. Sucrose from a 2 M stock solution made in hypotonic buffer is added to the cell lysate, resulting in a final concentration of 250 mM sucrose. This solution is mixed by inversion, and the nuclei are pelleted by centriguation at 400 x g in a swing out rotor for 10 to 40 minutes at 4°C. The supernatant is then discarded, and the pelleted nuclei are resuspended in 10-20 volumes of nuclear buffer (10 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 250 mM sucrose, 25 mM KC1, 1 mM DTT, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM PMSF). The nuclei are sedimented and resuspended in 1-2 volumes of nuclear buffer, as described above. The freshly isolated nuclei may either be used immediately for *in vitro* reprogramming and nuclear transfer into recipient cells or cytoplasts as described below or stored for later use. For storage, the nuclei are diluted in nuclear buffer to a concentration of approximately 10^6/ml. Glycerol (2.4 volumes of 100% glycerol) is added and mixed well by gentle pipetting. The suspension is aliquoted into 100-500 μl volumes in 1.5-ml tubes on ice, immediately frozen in a methanol-dry ice bath, and stored at -80°C. Prior to use, aliquots of the nuclei are thawed on ice or at room temperature. One volume of ice cold nuclear buffer is added, and the solution is centrifuged at 1,000 x g for 15 minutes in a swing-out rotor. The pelleted nuclei are resuspended in 100-500 μl nuclear buffer

and centrifuged as described above. The pelleted nuclei are then resuspended in a minimal volume of nuclear buffer and stored on ice until use.

Preparation of the reprogramming extract

Interphase cultured cells as harvested using standard methods and washed by centrifugation at 500 x g for 10 minutes in a 10 ml conical tube at 4°C. Preferably, the cells are of the desired cell type that one wishes the recipient cell or cytoplast to become. The supernatant is discarded, and the cell pellet is resuspended in a total volume of 50 ml of cold PBS. The cells are centrifuged at 500 x g for 10 minutes at 4°C. This washing step is repeated, and the cell pellet is resuspended in approximately 20 volumes of ice-cold interphase cell lysis buffer (20 mM Hepes, pH 8.2, 5 mM $MgCl_2$, 1 mM DTT, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, 100 μM PMSF, and optionally 20 μg/ml cytochalasin B). The cells are sedimented by centrifugation at 800 x g for 10 minutes at 4°C. The supernatant is discarded, and the cell pellet is carefully resuspended in no more than one volume of interphase cell lysis buffer. The cells are incubated on ice for one hour to allow swelling of the cells. The cells are lysed by either sonication using a tip sonicator or Dounce homogenization using a glass mortar and pestle. Cell lysis is performed until at least 90% of the cells and nuclei are lysed, which may be assessed using phase contrast microscopy. The sonication time required to lyse at least 90% of the cells and nuclei may vary depending on the type of cell used to prepare the extract.

The cell lysate is placed in a 1.5-ml centrifuge tube and centrifuged at 10,000 to 15,000 x g for 15 minutes at 4°C using a table top centrifuge. The tubes are removed from the centrifuge and immediately placed on ice. The supernatant is carefully collected using a 200 μl pipette tip, and the supernatant from several tubes is pooled and placed on ice. This supernatant is the "interphase cytoplasmic" or "IS15" extract. This cell extract may be aliquoted into 20 μl volumes of extract per tube on ice and immediately flash-frozen on liquid nitrogen and stored at -80°C until use. Alternatively, the cell extract is placed in an ultracentrifuge tube on ice (*e.g.*, fitted for an SW55 Ti rotor; Beckman). If necessary, the tube is overlayed with mineral oil to the top. The extract is centrifuged at 200,000 x g for three hours at 4°C to sediment membrane vesicles contained in the IS15 extract. At the end of

centrifugation, the oil is discarded. The supernatant is carefully collected, pooled if necessary, and placed in a cold 1.5 ml tube on ice. This supernatant is referred to as "IS200" or "interphase cytosolic" extract. The extract is aliquoted and frozen as described for the IS15 extract.

Reprogramming of nuclei in the extract

Either freshly isolated or thawed purified nuclei are resuspended in the reprogramming extract described in the previous section at a concentration of 4,000-5,000 nuclei/μl. An ATP generating system (1 mM ATP, 10 mM creatine phosphate, 25 μg/ml creatine kinase) and 100 μM GTP are added to the interphase extract to promote active uptake of nuclear components by the exogenous nuclei. The reaction is incubated at 30°C for up to two hours. Uptake of specific nuclear components may be monitored by immunofluorescence analysis of the nuclei.

Preferably, the factor is at least 75%, more preferably, at least 90%, and most preferably, at least 99%, by weight, pure. A substantially pure factor may be obtained by chemical synthesis, separation of the factor from natural sources, or production of the factor in a recombinant host cell that does not naturally produce the factor. Proteins, vesicles, chromosomes, nuclei, and other organelles may be purified by one skilled in the art using standard techniques such as those described by Ausubel *et al.* (Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2000). The factor is preferably at least 2, 5, or 10 times as pure as the starting material, as measured using polyacrylamide gel electrophoresis, column chromatography, optical density, HPLC analysis, or western analysis (Ausubel *et al., supra*). Preferred methods of purification include immunoprecipitation, column chromatography such as immunoaffinity chromatography, magnetic bead immunoaffinity purification, and panning with an immobilized antibody.

Purification of reprogrammed nuclei out of the extract

The reprogrammed nuclei are centrifuged at 1,000 x g for 10-30 minutes through a 1 M sucrose cushion prepared in nuclear buffer at 4°C. The nuclei are washed by resuspending them in 500 μl cold nuclear buffer and centrifuging at 1,000 x g for 10 minutes at 4°C. The nuclei are resuspended in nuclear buffer and

held on ice until use for nuclear transfer into the cytoplasm of recipient cells or cytoplasts.

Enucleation of recipient cells

Preferably, part or all of the DNA in the recipient cell is removed or inactivated. This destruction or removal of the DNA in the recipient cell prevents the genetic material of the cell from contributing to the characteristics and function of the reprogrammed cell. One method for destroying the nucleus of the cell is exposure to ultraviolet light (Gurdon, in *Methods in Cell Biology, Xenopus Laevis: Practical Uses in cell and Molecular Biology*, Kay and Peng, eds., Academic Press, California, volume 36:pages 299-309, 1991). Alternatively, the nucleus may be surgically removed by any standard technique (see, for example, McGrath and Solter, Science 220:1300-1319, 1983). In one possible method, a needle is placed into the cell, and the nucleus is aspirated into the inner space of the needle. The needle may then be removed from the cell without rupturing the plasma membrane (U.S. Patent Numbers 4,994,384 and 5,057,420).

Introduction of reprogrammed nuclei into recipient cells or cytoplasts

The nuclei are introduced into recipient cells or cytoplasts of either the desired cell type or of any other cell type using standard methods, such as microinjection or electrofusion (see, for example, U.S. Patent Numbers 4,997,384 and 5,945,577). The reconstituted cells are placed back in culture and allowed to recover, divide, and differentiate according to the reprogrammed pathway. Gene expression by the reprogrammed cells may be monitored using standard Northern analysis to measure expression of mRNA molecules, preferably mRNA molecules that are specific for the donor cell, recipient cell, or the desired cell type (Ausubel, *et al., supra*). Expression of specific mRNA molecules may also be detected using standard reverse-transcription polymerase chain reaction (RT-PCR) assays with primers designed to specifically bind an mRNA molecule of interest. Alternatively, the expression of multiple cell specific mRNA molecules may be monitored using standard DNA chip technology with cDNA arrays (Marrack *et al.*, Current Opinion in Immunology 12, 206-209, 2000; Harkin, Oncologist. 5:501-507, 2000; Pelizzari *et al.*, Nucleic Acids Res. 2;28(22):4577-4581, 2000; Marx, Science 289(5485):1670-1672, 2000). The cells may be analyzed for a reduction in

expression of genes specific for the cell type of the donor cell, recipient cell, or recipient cytoplast. Additionally, cells may be assayed for an increase in expression of genes specific for the desired cell type. Examples of mRNA molecules that are indicative of reprogramming to generate a stem cell include H-19, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCTM-2, Oct-4, Genesis, GCNF, GDF-3, and TDGF-1. Neural cell specific mRNA molecules include, but are not limited to, NGF, NF-H, NeuN, NSE, and CD11b. For analyzing the conversion to an adipocyte cell fate, expression of mRNA molecules such as leptin, PPARλ1, PPARλ2, SREBP1C, IR, and TNFα may be monitored. IGF-1 and IR are indicative of insulin producing cells. Additionally, the cells may be analyzed for expression of particular proteins using standard Western or immunofluorescence analysis (Ausubel *et al., supra*).

Examples of other characteristics of the reprogrammed cell that may be analyzed to determine whether it has been converted into the desired cell type include the size of the cell, cell morphology, ability to grow as an adherent cell, ability to grow as an attached cell, volume of cytoplasm, and location of a centrosome. The functions of the reprogrammed cells may also be tested, such as the ability of red blood cells to transport O_2 and CO_2, the ability of B-cells to make antibodies, and the ability of neutrophiles to phagocytose and destroy invading bacteria. Additionally, the production of lipids by adipocytes types may be determined using standard microscopy to visualize lipid droplets in the cells.

EXAMPLE 2

Two-step in vitro reprogramming method

In another method for reprogramming cells, nuclei are isolated from interphase cells and incubated in a mitotic extract, a detergent and salt solution, or a protein kinase solution to induce nuclear envelope breakdown and the formation of chromatin masses. This incubation causes the release of factors from the chromatin masses. Alternatively, chromatin masses may be isolated from mitotic cells. Preferably, the chromatin masses are then incubated in an interphase reprogramming extract to promote the formation of nuclear membranes and the addition of desired factors from the extract to the resulting nuclei. The reprogrammed nuclei are then isolated from the extract and inserted into recipient cells or cytoplasts of the desired cell type or of any other cell type.

Alternatively, the chromatin masses may be directly inserted into recipient cells or cytoplasts without first being induced to reform nuclei. For this embodiment, recipient cells or cytoplasts of the desired cell type are used so that desired factors from the cytoplasm of the recipient cells or cytoplasts may bind the exogenous chromosomes from the donor chromatin masses and further promote the expression of desired mRNA and protein molecules.

Preparation of mitotic cell extract

A mitotic cytoplasmic (MS15) or mitotic cytosolic (MS200) extract may be prepared as described above for interphase IS15 or IS200 extracts, except that mitotic cells are used instead of interphase cells and that 10 mM EDTA is added to the cell lysis buffer. For the isolation of mitotic cells, somatic cells are synchronized in mitosis by incubating them in 0.5-1 μg/ml nocodazole for 17-20 hours, and the mitotic cells are detached by vigorous shaking, as described above. The detached G_1 phase doublets may be discarded, or they may be allowed to remain with the mitotic cells which constitute the majority (over 80%) of the detached cells. The harvested detached cells are centrifuged at 500 x *g* for 10 minutes in a 10 ml conical tube at 4°C.

Chromosome condensation reaction in mitotic extract for removal of endogenous nuclear components

An aliquot of MS15 or MS200 extract is thawed on ice. An ATP-generating system (0.6 μl) and GTP are added to 20 μl of extract and mixed by vortexing, resulting in final concentrations of 1 mM ATP, 10 mM creatine phosphate, 25 μg/ml creatine kinase, and 100 μM GTP.

Nuclei are isolated from donor cells as described above. The nuclei suspension is added to the extract at a concentration of 1 μl nuclei per 10 μl of extract, mixed well by pipetting, and incubated in a 30, 33, 35, 37, or 39°C water bath. The tube containing the mixture is tapped gently at regular intervals to prevent chromosomes from clumping at the bottom of the tube. Nuclear envelope breakdown and chromosome condensation is monitored at regular intervals, such as every 15 minutes, under a microscope. When the nuclear envelope has broken down

and chromosomes have started to condense, the procedure for recovery of chromatin masses from the extract is started.

Formation of decondensed chromatin masses by exposure of nuclei to mitotic extract and anti-NuMA antibodies

Alternatively, chromatin masses that are not condensed or only partially condensed may be formed by performing the above procedure after pre-loading the isolated nuclei with an antibody to the nuclear matrix protein NuMA (Steen *et al.*, J. Cell Biol. 149, 531-536, 2000). This procedure allows the removal of nuclear components from chromatin by the dissolution of the nuclear membrane surrounding the donor nuclei; however, the condensation step is inhibited by addition of the anti-NuMA antibody. Preventing chromosome condensation may reduce the risk of chromosome breakage or loss while the chromosomes are incubated in the mitotic extract.

For this procedure, purified cell nuclei (2,000 nuclei/μl) are permeabilized in 500 μl nuclear buffer containing 0.75 μg/ml lysolecithin for 15 minutes at room temperature. Excess lysolecithin is quenched by adding 1 ml of 3% BSA made in nuclear buffer and incubating for 5 minutes on ice. The nuclei are then sedimented and washed once in nuclear buffer. The nuclei are resuspended at 2,000 nuclei/μl in 100 μl nuclear buffer containing an anti-NuMA antibody (1:40 dilution; Transduction Laboratories). After a one-hour incubation on ice with gentle agitation, the nuclei are sedimented at 500 x g through 1 M sucrose for 20 minutes. The nuclei are then resuspended in nuclear buffer and added to a mitotic extract containing an ATP regenerating system, as described in the previous section. Optionally, the anti-NuMA antibody may be added to the extract to further prevent chromosome condensation.

Formation of decondensed chromatin masses by exposure of nuclei to a detergent or protein kinase

Chromatin masses that are not condensed or only partially condensed may also be formed by exposure to a detergent or protein kinase. A detergent may be used to solubilize nuclear components that are either unbound or loosely bound to the chromosomes in the nucleus, resulting in the removal of the nuclear envelope.

For this procedure, purified cell nuclei (2,000-10,000 nuclei/μl) are incubated in nuclear buffer supplemented with a detergent, such as 0.1% to 0.5% Triton X-100 or NP-40. To facilitate removal of the nuclear envelope, additional salt, such as NaCl, may be added to the buffer at a concentration of approximately 0.1, 0.15, 0.25, 0.5, 0.75, or 1 M. After a 30-60 minute incubation on ice with gentle shaking, the nuclei are sedimented by centrifugation at 1,000 x g in a swing-out rotor for 10-30 minutes, depending on the total volume. The pelleted nuclei are resuspended in 0.5 to 1 ml nuclear buffer and sedimented as described above. This washing procedure is repeated twice to ensure complete removal of the detergent and extra salt.

Alternatively, the nuclear envelope may be removed using recombinant or naturally-occurring protein kinases, alone or in combination. The protein kinases may be purified using standard procedures or obtained in purified form from commercial sources. These kinases may phosphorylate components of the nuclear membrane, nuclear matrix, or chromatin, resulting in removal of the nuclear envelope (see, for example, Collas and Courvalin, Trends Cell Biol. 10: 5-8, 2000). Examples of kinases include cyclin-dependent kinase 1 (CDK1), protein kinase C (PKC), protein kinase A (PKA), MAP kinase, and calcium/calmodulin-dependent kinase (CamKII). For this method, approximately 20,000 purified nuclei are incubated in 20 μl of phosphorylation buffer at room temperature in a 1.5 ml centrifuge tube. A phosphorylation buffer for CDK1 (Upstate Biotechnology) may contain 200 mM NaCl, 50 mM Tris-HCl (pH 7.2-7.6), 10 mM MgS0, 80 mM β-glycerophosphate, 5 mM EGTA, 100 μM ATP, and 1 mM DTT. For PKC, for example, the buffer may contain 200 mM NaCl, 50 mM Tris-HCl (pH 7.2-7.6), 10 mM $MgSO_4$, 100 μM $CaCl_2$, 40 μg/ml phosphatidylserine, 20 μM diacylglycerol, 100 μM ATP, and 1 mM DTT. If both PKC and CDK1 are used simultaneously, the CDK1 phosphorylation buffer supplemented with 40 μg/ml phosphatidylserine and 20 μM diacylglycerol is used. An example of a phosphorylation buffer for PKA includes 200 mM NaCl, 10 mM $MgSO_4$, 10 mM Tris, pH 7.0, 1mM EDTA, and 100 μM ATP. For MAP kinase, the PKA phosphorylation buffer supplemented with 10 mM $CaCl_2$, and 1 mM DTT may be used. For CamKII, either PKA buffer supplemented with 1 mM DTT or a Cam Kinase assay kit from Upstate Biotechnology (Venema *et al.* J. Biol. Chem 272: 28187-90, 1997) is used.

The phosphorylation reaction is initiated by adding a protein kinase to a final amount of 25-100 ng. The reaction is incubated at room temperature for up to one hour. Nuclear envelope breakdown may be monitored by microscopy during this incubation, such as at 15-minute intervals. After nuclear envelope breakdown, nuclei are washed three times, as described above for the removal of the detergent solution.

Recovery of chromatin masses from the extract, detergent and salt solution, or protein kinase solution

The extract or solution containing the condensed, partially condensed, or not condensed chromatin masses is placed under an equal volume of 1 M sucrose solution made in nuclear buffer. The chromatin masses are sedimented by centrifugation at 1,000 x g for 10-30 minutes depending on the sample volume in a swing out rotor at 4°C. The supernatant is discarded and the pelleted chromatin masses are carefully resuspended by pipetting in 0.1-1.0 ml nuclear buffer and centrifuged at 1,000 x g for 10-30 minuets. The supernatant is discarded, and the pelleted chromatin masses are resuspended in nuclear buffer and stored on ice until use.

Isolation of chromatin masses from mitotic cells

As an alternative to generating chromatin masses by exposure of nuclei to a mitotic extract, a detergent and salt solution, or a protein kinase solution, chromatin masses may be obtained by lysis of cells synchronized in mitosis and centrifugation of the cell lysate as described herein.

Preparation of membrane vesicles for nuclear reassembly *in vitro*

The pellet generated from the 200,000 x g centrifugation during the preparation of the MS2000 mitotic extract is used as a source of mitotic membrane vesicles. This pellet is resuspended in membrane wash buffer (250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 50 mM Hepes, pH 7.5, 1 mM DTT, 1 mM ATP, 10 μM aprotinin, 10 μM leupeptin, 10 μM pepstatin A, 10 μM soybean trypsin inhibitor, and 100 μM PMSF), centrifuged at 100,000 x g for 30 minutes, aliquoted, frozen in liquid nitrogen, and stored at -80°C.

Nuclear reassembly assay

If desired, nuclei may be reassembled from condensed, partially condensed, or decondensed chromatin masses as described below. The reformation of the nuclear membrane around the chromosomes may encapsulate factors from the extract used for reassembly allowing them to be transferred as part of the reformed nucleus into the recipient cell or cytoplast. The chromatin masses are recovered by sedimentation through a 1.0 M sucrose cushion and are resuspended in interphase extract at a concentration of 4,000 - 5,000 chromatin masses/μl. This interphase extract may be formed from cells of the cell type that is desired, as described above. The extract is supplemented with membrane vesicles prepared as described above to provide membranes which are required for nuclear evelope assembly. The membranes are added at a concentration of 1.0 μl thawed membranes per 10 μl extract and mixed by vortexing. And ATP generating system (2 mM ATP, 20 mM creatine phosphate, 50 μg/ml creatine kinase) and 100 μM GTP are added to the interphase extract to promote chromatin decondensation, binding of nuclear membrane vesicles to chromatin, and vesicle fusion to form an intact nuclear membrane. The reaction is incubated at 30°C for up to two hours, and nuclear reassembly is monitored by phase contract microscopy.

Purification of reprogrammed nuclei out of the extract

Reprogrammed nuclei are centrifuged at 1,000 x g for 10-30 minutes through a 1.0 M sucrose cushion prepared in nuclear buffer at 4°C. The nuclei are washed by resuspending them in 500 μl cold nuclear buffer and sedimentation at 1,000 x g for 10 minutes at 4°C. Then, the nuclei are resuspended in nuclear buffer and held on ice until use for nuclear transfer into the cytoplasm of recipient cells or cytoplasts.

Introduction of reprogrammed nuclei or chromatin masses into recipient cells or cytoplasts

The chromatin masses or nuclei formed from the chromatin masses are inserted into recipient cells or cytoplasts using standard methods, and gene expression is monitored, as described above.

EXAMPLE 3

Reprogramming of permeabilized cells without nuclear transfer

Cells may also be reprogrammed without requiring the isolation of nuclei or chromatin masses from the cells. In this method, interphase or mitotic cells are permeabilized and then incubated in an interphase or mitotic reprogramming extract under conditions that allow the exchange of factors between the extract and the cells. If an interphase extract is used, the nuclei in the cells remain membrane-bounded; if a mitotic extract is used, nuclear envelope breakdown and chromatin condensation may occur. After the nuclei are reprogrammed by incubation in this extract, the plasma membrane may be resealed, forming an intact reprogrammed cell that contains desired factors from the extract.

Permeabilization of cells

Cells that may be reprogrammed using this procedure include unsynchronized cells and cells synchronized in G_0, G_1, S, G_2, or M phase, cells arrested in the cell cycle by natural differentiation or by exogenous agents that arrest the cell cycle, senescence, or a combination of these. The cells are permeabilized using any standard procedure, such as permeabilization with digitonin or Streptolysin O. Briefly, cells are harvested using standard procedures and washed with PBS. For digitonin permeabilization, cells are resuspended in culture medium containing digitonin at a concentration of approximately 0.001 - 0.1% and incubated on ice for 10 minutes. For permeabilization with Streptolysin O, cells are incubated in Streptolysin O solution (see, for example, Maghazachi *et al.*, 1997 and references therein) for 15-30 minutes at room temperature. After either incubation, the cells are washed by centrifugation at 400 x g for 10 minutes. This washing step is repeated twice by resuspension and sedimentation in PBS. Cells are kept in PBS at room temperature until use. The cells can then be immediately added to the interphase or mitotic extract for reprogramming, as described below.

Reprogramming of cells in an extract

An interphase or mitotic extract is prepared as described above, for example, using cells of the cell type that one desires the premeabilized cells to become. The permeabilized cells are suspended in the reprogramming extract at a concentration of

approximately 100-1,000 cells/μl. The ATP generating system and GTP are added to the extract as described above, and the reaction is incubated at 30-37°C for up to two hours to promote translocation of factors from the extract into the cell and active nuclear uptake or chromosome-binding of factors. The reprogrammed cells are centrifuged at 800 x g, washed by resuspension, and centrifugation at 400 x g in PBS. The cells are resuspended in culture medium containing 20-30% fetal calf serum (FCS) and incubated for 1-3 hours at 37°C in a regular cell culture incubator to allow resealing of the cell membrane. The cells are then washed in regular warm culture medium (10% FCS) and cultured further using standard culturing conditions.

Example 3: Reprogramming using an activated T-cell extract

Cell extracts from activated human T-cells were used to induce nuclear localization of transcription factors in unactivated human T-cells, B-cells, human fibroblast, and HeLa cells. Additionally, this incubation promoted DNA-binding of the chromatin remodeling SWI/SNF complex, hyperacetylation of the IL2 gene, and expression of IL2 mRNA in unactivated T-cells.

In order to demonstrate that activation of intact T-cells induces expression of IL2, human T-cells can be purified from peripheral blood, cultured overnight, and stimulated with anti-CD3 antibodies. At the indicated time points, cells are diluted with ice-cold PBS, snap-frozen in liquid nitrogen, thawed, and washed in PBS. Total RNA is isolated, and RT-PCR was performed using IL2-specific primers. IL2 mRNA is expressed in activated T-cells but not expressed in mock-treated T-cells ("30c," "60c," and "120c" denote mock-treated cells).

In order to determine whether an extract from activated T-cells can increase nuclear localization of transcription factors in other cells, T-cells can be activated by incubation in the presence of an anti-CD3 antibody, and then the cells are washed to remove the unbound antibody. A stimulated T-cell extract is prepared by lysing these T-cells, centrifuging them at 15,000 x g, isolating the supernatant, and adding the ATP-generating system to the supernatant. Nuclei purified from resting T-cells, from the B-cell line Reh, 293T fibroblasts, of HeLa cells are incubated in this extract for 30 minutes at the concentration of approximately 5,000 nuclei per μl of extract. Then, the nuclei are sedimented through a sucrose cushion. Immunofluorescence

analysis can demonstrate the T-cell specific transcription factor NFAT is imported into the nuclei exposed to the stimulated extract.

The ability of other transcription factors from the extract to migrate into the nuclei of T-cells, B-cells, fibroblasts, and HeLa cells can also be determined. For this assay, input nuclei ("Input") from unstimulated T-cells are incubated in either stimulated extract ("SE"), control extract prepared from unstimulated T-cells (denoted "USE" for unstimulated extract), or stimulated extract containing a monoclonal antibody against nucleoporins which sterically blocks nuclear import ("SE + mAb414"). The nuclei are then purified from the extract by centrifugation and resuspension. It can be demonstrated that T-cell nuclei incubated in the stimulated extract have increased levels of NFAT, c-Jun/AP1, NFkB, and MAP kinase (Erk1 and Erk2), as measured using standard Western blot analysis with an anti-histone H4 antibody as a loading control. In contrast, incubation of the nuclei in the unstimulated extract has negligible effect on the level of these transcription factors. Additionally, NFAT, c-Jun/AP1, and NFkB levels are increased in nuclei from Reh B-cells, 293T fibroblasts, and HeLa cells after incubation in the stimulated T-cell extract.

For T-cell nuclei exposed to either the stimulated or unstimulated extract, DNA-binding by these transcription factors was assessed using a standard nuclear retention assay. This assay involves extraction of nuclei with 0.1% Triton X-100 to dissolve the nuclear membrane and sedimentation at 15,000 x g. Immunoblot analysis is performed on the pellet, which contains transcription factors that are bound to DNA, and the supernatant, which contains the unbound transcription factors. The percentage of DNA-bound transcription factors can be determined by densitometric analysis of duplicate blots. This demonstrates the increased nuclear import and DNA-binding of NFAT, c-Jun/AP1, NFkB, and MAP kinase transcription factors in reprogrammed T-cell nuclei.

For demonstration of the effect of exposing T-cell nuclei to the stimulated extract on the DNA-binding of the chromatin remodeling SWI/SNF complex, resting T-cell nuclei can be incubated in cell lysis buffer, the unstimulated extract, or the stimulated extract for 30 minutes and sedimented through sucrose. The percentage of DNA-bound and unbound SWI/SNF after various incubation times is assessed using the above nuclear retention assay with anti-BRG1 antibodies to visualize the SWI/SNF complex. Exposure of the nuclei to the stimulated extract increases the

amount of DNA-bound SWI/SNF, suggesting that reprogramming of the nuclei is occurring.

To measure hyperacetylation of the IL2 gene of T-cell nuclei *in vivo* and *in vitro*, micrococcal nuclease is used to digest the chromatin from resting T cells, anti-CD3 stimulated T-cells, T-cell nuclei exposed to an unstimulated extract, and T-cell nuclei exposed to a stimulated extract, forming soluble chromatin fragments. Acetylated histone H4 ("H4ac") is immunoprecipitated from the soluble chromatin fraction, and DNA is isolated from immune precipitate ("bound") and supernatant ("unbound") fractions. The DNA is dot-blotted on duplicate Hybon N filters and hybridized to either a fluoresceinated IL2 or carp β-actin probe. Hybridization is detected using alkaline phosphatase-conjugated anti-fluorescein antibodies. Hyperacetylation of the IL2 gene, but not the control β-actin gene, is observed in nuclei exposed to the stimulated extract, further suggesting that the nuclei were being reprogrammed to express genes usually repressed by the nuclei.

To demonstrate the ability of the stimulated extract to induce expression of IL2, resting T-cell nuclei are incubated for 30 minutes at 30°C in unstimulated extract or stimulated extract. As controls, nuclei are incubated in stimulated extract containing either 100 μg/ml RNAse A, 100 μg/ml DNAse I, mAb414 antibodies, or the lectin WGA. After 30 minutes at 30°C, nuclei are lysed in the extracts by sonication and 3 μl extract aliquots are removed for RT-PCR analysis using IL2-specific primers. Input stimulated extract and a control stimulated extract containing 1.2 μg total RNA isolated from IL2-producing T-cells are then analyzed. This demonstrates that IL2 expression is induced by incubation of T-cell nuclei in the stimulated extract but not by incubation in any of the control extracts. This *in vitro* IL2 mRNA production is dependent on PolII transcription. For this assay, nuclei are exposed for 30 minutes to stimulated extract containing increasing concentrations of the RNA PolII inhibitor actinomycin D (0, 5, 10, 50, 100 and 500 nM), and IL2 mRNA synthesis is analyzed by RT-PCR. As a control, extracts from anti-CD3 stimulated B-cells, fibroblasts, and HeLa cells, which do not express IL2, are tested for their ability to induce IL2 expression in nuclei from resting T-cells.

Other Embodiments

From the foregoing description, it will be apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.